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FULL PAPER

Applying a thermostable and organic solvent tolerant ene-reductase for the asymmetric reduction of (*R*)-carvone

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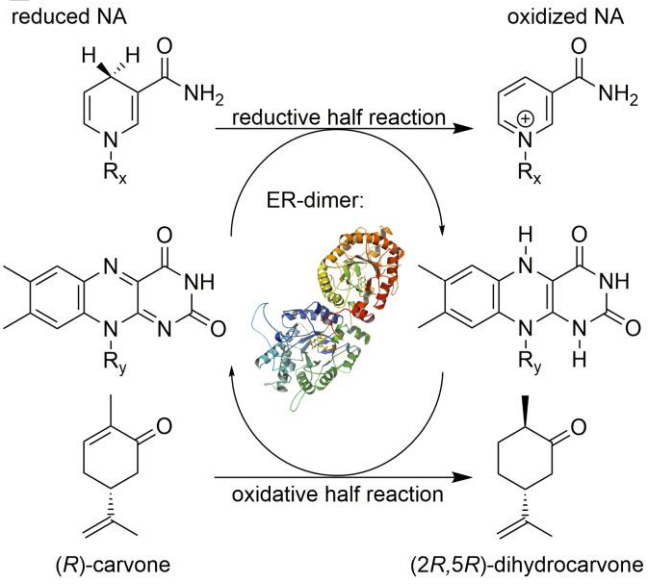
Abstract: Ene-reductases allow regio- and stereoselective reduction of activated C=C double bonds at the expense of nicotinamide adenine dinucleotide cofactors (NAD(P)H). Biological NAD(P)H can be replaced by synthetic mimics to facilitate enzyme screening and process optimization. The ene-reductase *F*OYE-1, originating from an acidophilic iron oxidizer, has been described as a promising candidate and is now explored for applied biocatalysis. Biological and synthetic nicotinamide cofactors were evaluated to fuel *F*OYE-1 to produce valuable compounds. A maximum activity of 319.7 ± 3.2 U mg^{-1} with NADPH or of 206.7 ± 3.4 U mg^{-1} with 1-benzyl-1,4-dihydronicotinamide (BNAH) for the reduction of *N*-methylmaleimide was observed at 30 °C. Especially, BNAH was found to be a promising reductant but exhibits poor solubility in water. Different organic solvents were assayed and *F*OYE-1 showed excellent performance in most systems with up to 20 vol% solvent and up to 40 °C. Purification and application strategies were evaluated on a small scale to optimize the process. Finally, a 200 mL biotransformation of 750 mg (*R*)-carvone afforded 495 mg of (2*R*,5*R*)-dihydrocarvone (>95% *ee*), demonstrating the simplicity to handle and apply *F*OYE-1.

Introduction

The monoterpenoid (2*R*,5*R*)-dihydrocarvone represents a chiral building block to synthesize natural products, such as sesquiterpenes or striatenic acid, co-polymers, and antimalarial drugs.[1-3] It is naturally present in dill oil and caraway seeds, but cannot be obtained *via* extraction.[4] On a large scale, it is produced by either carvone hydrogenation or limonene oxidation.[3] Due to its industrial relevance, its production through several strategies was investigated.[2,3,5] Among those strategies, the use of biocatalysts has been described and here the ene-reductases (ERs) acting on (*R*)-carvone are preferred (Scheme 1).[6] Despite the catalytic power and selectivity of those enzymes, employing ERs as biocatalysts brings several challenges which need to be addressed.[5b,6] First, these enzymes require reducing power that is naturally obtained from NAD(P)H. This cofactor can be recycled *in situ* in whole-cell and cell-free systems[2,7] or can be replaced by nicotinamide cofactor mimics in cell-free systems.[6d,8] Second, in this case the substrate and product (carvone and dihydrocarvone) are poorly soluble in water and thus co-solvents are necessary and need to be implemented in the system used.[9] These co-solvents act as a substrate reservoir and as an extraction solvent for *in situ* product removal. Third, carvones are reported to have toxic or antimicrobial activity,[10] which limits the application of whole-cell biotransformation without further process engineering.[2,9] Finally, ER-based whole-cell biotransformation can result in by-products caused by racemization.[2] Therefore, cell-free ER approaches in combination with a simple source of reducing power might be favored for small scale studies in order to rapidly obtain highly pure products. Herein the cost effective 1-benzyl-1,4-dihydronicotinamide (BNAH) shall be used.



Scheme 1. Stereoselective reduction of (*R*)-carvone by an ene-reductase (ER). The nicotinamide (biological or synthetic; NA) acts as electron donor to reduce the flavin cofactor FMN (flavin mononucleotide) of ERs which subsequently allows to transfer a hydride to C_α of the unsaturated substrate. A proton from a conserved Tyr in ER is added to C_β to yield (2*R*,5*R*)-dihydrocarvone.[2,6]

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Ene-reductases of the Old Yellow Enzyme family (OYEs) are flavin-dependent enzymes employing nicotinamide cofactors for an initial flavin reduction followed by a direct *trans*-hydrogenation of the substrate. A classic example would be the reduction of (*R*)-carvone to (2*R*,5*R*)-dihydrocarvone (Scheme 1).[2,6,9,11,12] The substrates of ERs typically require an activated C=C double bond that can be hydrogenated, generating up to two stereogenic centers.[6] To study and evaluate ERs, maleimides were employed first as these are cost-effective, soluble in buffer and do not interfere with the co-substrate or cofactor. However, it was shown that they can form covalent adducts with cysteine residues frequently found in the active site of ERs.[13] This leads to a rapid inactivation and can be circumvented either by site-directed mutagenesis or by avoiding enzyme incubation in presence of those maleimides.

Thermo- and solvent-stability are important factors to consider if an enzyme shall be employed in industrial biocatalysis.[6,9,13,14] Here, the above-mentioned substrate-based inactivation due to covalent modification should be avoided. Additionally, the enzyme must be stable over time to allow high turnover numbers and increased rates by higher reaction temperatures and/or the possibility to add co-solvents. The use of co-solvents is furthermore important for solubilization of the (co)substrates carvone and 1-benzyl-1,4-dihydronicotinamide (BNAH), for example.

In this study we describe the recently identified thermostable ene-reductase *F*OYE-1[14b] with respect to applied biocatalysis in order to show its potential to produce an optically pure compound of industrial interest, (*2R*,*5R*)-dihydrocarvone.[1-5,9-11] This procedure provides access towards chiral and highly pure ER-products as well as to simple-to-handle and scalable biotransformations. The activity of *F*OYE-1 is described for the first time with an efficient usage of BNAH. In addition, the enzymatic activity and stability in presence of various organic solvents was investigated.

Results and Discussion

Enzyme production and specific activity. The protein *F*OYE-1 was produced in a similar yield as described before, 4.2 mg per L fermentation broth in a non-optimized gene expression.[14b] Despite indications for the formation of inclusion bodies (Fig. S1), the total amount (8.4 mg from 2 L expression culture), allowed to investigate catalytic properties and stability issues as well as to set up various biotransformations.

In the first place, the protein was purified by Ni-chromatography and total activity was determined as described previously.[14b] The specific activity of the enzyme [U mg^{-1}] is by convention given as the rate of substrate consumption or respective product formation [µmol min^{-1}] per mg enzyme. This amounted to a specific activity of 60.8 U mg^{-1} at standard conditions (50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.1, 1 mM maleimide **1**, 200 µM NADPH; NADPH consumption) with respect to the total protein amount of the preparation. This is in congruence to earlier investigations (65.4 U mg^{-1}) and showed the reproducibility of *F*OYE-1 production. The chromatographically enriched protein was found to be only partially saturated with the flavin mononucleotide cofactor FMN (46.5% of monomer loaded with FMN for the batch used for most experiments herein presented). Protein production was repeated three times and the obtained purified protein showed FMN saturations in the range of 30 to 50%, respectively. Loading the remaining apoprotein by addition of excess FMN, followed by incubation and washing steps to remove surplus free FMN, was partially successful. In case of excess FMN over total protein the reaction rate could be increased by a factor of 1.3 to 1.7 in dependency of the protein batch. Respectively, a maximum FMN load of 60 to 80% of purified *F*OYE-1 was reached while the specific activity remained stable. Thus, only a fraction of the total protein pool participated in catalysis and therefore specific activity calculations in this study are generally based on the amount of protein with FMN-saturated active sites. In addition, it needs to be mentioned that surplus FMN in solution can be reduced by the BNAH employed for biocatalysis. This may lead to side reactions such as unproductive hydrogen peroxide formation or $FMNH_2$-protein interaction and make any kinetic data analysis or turnover calculations complicated.

Table 1. Observed activity of *F*OYE-1 on various substrates (Fig. S3).

Nr.	Substrate	Temp. [°C] [a]	Observed activity [U mg^{-1}] [b]
1	maleimide	20	130.7 ± 1.6
2	*N*-methylmaleimide	20	144.7 ± 3.7
		22.5	172.0 ± 18.0
		30	264.1 ± 2.2
3	*N*-(2,4,6-trichlorophenyl)maleimide	22.5	11.6 ± 0.3
4	indole-2-carboxylic acid	22.5	n.d.
5	3-hydroxy-2-methyl-4-pyrone	22.5	n.d.
6	mesaconic acid	22.5	0.7 ± 0.1

[a] Temperature was set to ambient conditions and kept constant during the assays. [b] The assay comprised 50 mM KH_2PO_4/Na_2HPO_4 buffer (pH 7.1), 1 mM (compounds **1**-**3**) or 10 mM (compounds **4**-**6**) substrate, 200 µM NADPH, and 30 nM purified *F*OYE-1, no additional FMN. The activity was calculated based on FMN-saturated *F*OYE-1. n.d. = not detected.

FMN-saturated *F*OYE-1 (holoprotein) had an observed specific activity of 130.7 U mg^{-1} on maleimide **1** with NADPH as electron donor (Table 1). This calculation procedure was used for all subsequent activity data. The kinetic data fit to a clear kinetic according to the Michaelis-Menten model as it is typical for ERs.[6,8a,15] A number of potential substrates was screened and compared to the standard (Table 1, Fig. S3). It was proven that maleimides are preferred substrates of class III ERs,[6d,14] and especially *N*-methylmaleimide **2** was converted efficiently.

*F*OYE-1 was prepared and purified under different conditions to establish a simple preparation for biotransformation studies (Table S1, Fig. S2). It became clear that either the direct use of crude extract or purified protein should be preferred: Without substantial purification, the specific activity of *F*OYE-1 is already roughly 62% of the maximum reachable activity achieved by affinity chromatography, making this a potentially economic preparation for scaled-up work.

With a partial FMN saturation of 46.5%, the remaining apo-*F*OYE-1 in the cell free crude extract could be saturated with FMN and add to the pool of functional protein. To check this, the assays were repeated in the presence of additional FMN (10 to 70 µM) and resulted in an increase in units of active enzyme while keeping the high specific activity. The maximum achievable FMN saturation of about 80% was already reached in presence of 10 µM FMN in the respective assays. With maleimide **1** as substrate, the observed activity increased from 64.5 mU to 109.7 mU (specific activity of 172 U mg^{-1}) at 22 °C and from 99.0 mU to 168.3 mU (264 U mg^{-1}) at 30 °C, respectively. Thus it can be stated that *F*OYE-1 is one of the most active OYE reported for this substrate so far.[6] Nevertheless, it needs to be mentioned that the surplus FMN was most likely not tightly bound to the protein, as a maximum saturation of only about 50% FMN could be determined in the purified *F*OYE-1 (cf. above).

Application of various electron donors. ERs are known to accept a variety of electron donors to reduce FMN, such as natural and synthetic nicotinamide cofactors (Scheme 1).[6d,8,17] *F*OYE-1 accepts both NADH and NADPH, with a clear preference for the phosphorylated form (Table 2).[14b]

Table 2. Performance of *F*OYE-1 with natural and artificial nicotinamide cofactors.

Electron donor [a]	Temperature [°C]	Observed activity [U mg^{-1}] [b]		
Conc. of electron donor [µM]		200	300	1000
NADH	20	7.3 ± 0.7	8.3 ± 0.3	8.6 ± 0.2
NADPH	20	140.5 ± 0.4	141.5 ± 2	163.1 ± 2.4
BNAH	20	76.0 ± 1.2	93.6 ± 1.6	174.1 ± 2.7
NADH	30	11.4 ± 0.5	11.6 ± 0.6	12.7 ± 1.0
NADPH	30	264.1 ± 2.2	282.2 ± 2.9	319.7 ± 3.2
BNAH	30	87.9 ± 2.2	108.9 ± 1.3	206.7 ± 3.4

[a] The electron donor served as the substrate to initiate the reaction. [b] The assay comprised 50 mM KH_2PO_4/Na_2HPO_4 buffer (pH 7.1), 1 mM **2**, 200 / 300 / 1000 µM of respective electron donor, and 8.6 nM *F*OYE-1 (holoprotein).

With respect to the cost factor, the traditional synthetic cofactor mimic BNAH is an attractive electron donor[6b,8] since it can be easily obtained at comparably low cost (about 12.50€ per g at 95% purity; 09/2019; compared to NADPH with about 615.00€ per g at 95% purity; 09/2019). Thus, we studied the kinetics and solvent stability of *F*OYE-1 employing BNAH since the latter is only partially soluble in aqueous buffers. In a first standardized test, BNAH was compared with NADH and NADPH at 20 and 30 °C (Table 2). NADPH was found to be the best electron donor, especially at elevated temperatures. About 3 to 5 times more BNAH is required to achieve similar activities.

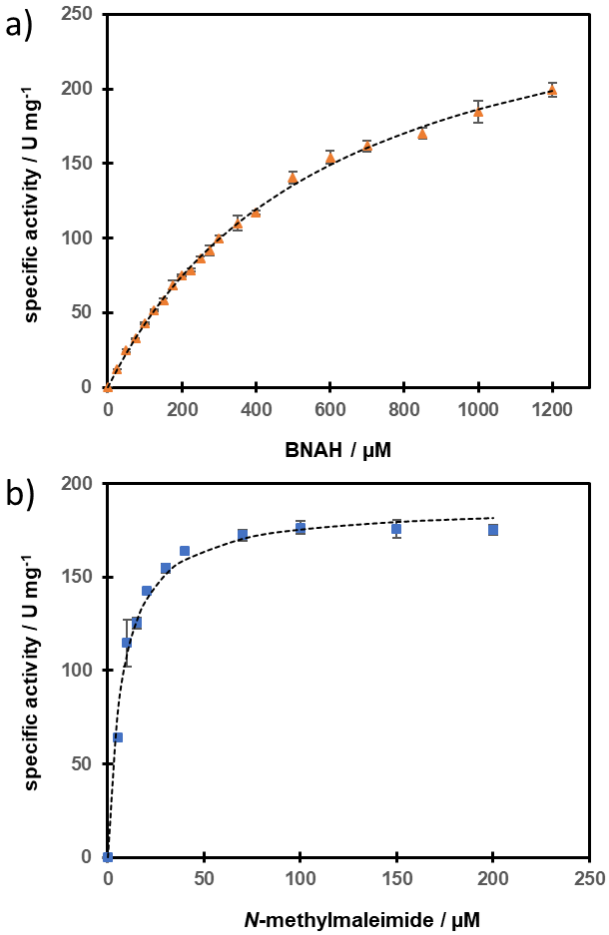

Figure 1. Kinetic analysis of *F*OYE-1 according to Michaelis-Menten with BNAH as cosubstrate. The standard enzyme assay was performed as described in the experimental section in KH_2PO_4/Na_2HPO_4 buffer at 22.5°C. 8.6 nM (0.375 µg mL^{-1}, holoprotein) *F*OYE-1 was used without the addition of extra FMN. The BNAH (a) or substrate **2** (b) concentration was varied (BNAH: 0-1200 µM and **2**: 0-200 µM) while the other was kept in excess. Data were analysed by non-linear fitting of the Michaelis-Menten equation by means of the KaleidaGraph software package (see Table 3).

The enzyme kinetics employing BNAH as the electron donor and *N*-methylmaleimide as main substrate (Fig. 1, Table 3) confirmed our assumption: more than 1 mM BNAH is needed to saturate the active site of *F*OYE-1 due to the high K_m value of about 600 µM. By comparing the kinetic parameters from the experiments varying either electron donor or substrate concentration while keeping the other constant, it became obvious that *F*OYE-1 suffers from non-productive uncoupling: The enzyme can employ only about 63% of the reducing equivalents of BNAH to reduce *N*-methylmaleimide. The latter is efficiently bound by the enzyme and converted at a high activity of about 188.6 U mg^{-1} at standard conditions (pH 7.1 and 22°C). Interestingly, the catalytic turnover frequencies are the same or even higher with mimics compared with NADPH, and only the ER XenA showed a comparable catalytic efficiency.[6d,14b,17]

Knaus *et al.* determined the steady-state kinetics for three ERs, namely PETNR, TOYE and XenA.[17] There, the enzyme performance with the natural nicotinamide electron donors compared to synthetic mimics was determined under substrate saturation (2-cyclohexen-1-one) at 30 °C. Similar experiments were performed with OYE*Ro*2a using *N*-methylmaleimide **2** as the substrate at 25 °C.[13a] From these data sets, the following order of catalytic efficiency can be drawn (under substrate saturation and with BNAH as electron donor): XenA (~1860 s^{-1} mM^{-1}) > *F*OYE-1 (~20%) > OYE*Ro*2a (~17%) > PETNR ≈ TOYE (4%). With respect to turnover frequency, *F*OYE-1 is the most powerful ER thereof ($k_{apparent}$ 4 to 40-times higher compared to the other ERs listed).[13a,17]

When adding 20 vol% acetone during kinetic experiments to enhance BNAH solubility (Table 3), the activity decreased by a factor of 5 and also the affinity for the cosubstrate was lowered significantly (factor of 2). Thus it might be reasoned that the co-solvent affects the protein structure or even its stability, thus limiting its applicability. The effect of organic solvents on the performance of *F*OYE-1 needed to be further elucidated (see below).

Table 3. Kinetic parameters of *F*OYE-1 towards BNAH and *N*-methyl-maleimide.

Substrate[a]/ Condition	K_m [µM]	V_{max} [U mg^{-1}]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} mM^{-1}]
BNAH	600 ± 19	298 ± 5	216 ± 4	360
BNAH/ 20 % acetone[b]	1133 ± 80	59.8 ± 2.7	43.4 ± 2	38
N-methyl-maleimide **2**	7.3 ± 0.7	188.6 ± 3.5	136.7 ± 2.5	18,726

The standard enzyme assay as described in the experimental section was performed in KH_2PO_4/Na_2HPO_4 buffer at 22.5°C. 8.6 nM (0.375 µg mL^{-1}, holoprotein) *F*OYE-1 was used without the addition of extra FMN. [a] The substrate concentration was varied (BNAH: 0-1200 µM and **2**: 0-200 µM) while the other one was kept in excess accordingly. [b] The same experiment was repeated in presence of 20 vol% acetone.

To evaluate the cosubstrate and its effect on biocatalysis, small-scale biotransformations were performed. A number of substrates was tested to demonstrate the scope and selectivity with respect to various electron donors (Fig. S3, Table S2). Varying the electron donor did not influence the enzyme's selectivity: In all cases the same enantiomers or diastereomers were obtained, mostly with a similar *ee*-value. The determined *ee*-values were generally acceptable, with substrates 2-methyl-*N*-phenylmaleimide **9**, (*R*)-carvone **12**, (*S*)-carvone **13**, dimethylcitraconic acid **14** and dimethylitaconic acid **16** converting with an *ee* above 90% (for substrate structures see Fig. S3). Products obtained at higher turnover frequencies can racemize in solution over time and thus gave moderate (**10**) to low (**7**) *ee* values with the natural electron donor and gave slightly lower enantiopurity with the synthetic electron donors, possibly due to even higher turnover frequencies.[17] With respect to conversion no significant difference was observed. It seems that in general, mimics can efficiently replace the natural electron donor (NADPH) for *F*OYE-1. Strikingly, (*R*)-carvone **12** was converted in a very selective way (97% optical purity, 14-18 % conversion) and thus became the model substrate for further investigations.

Activity and stability of *F*OYE-1 in presence of organic solvents. When employing non-natural nicotinamide mimics and various organic substrates, the use of co-solvents in order to bring both to the active site of the enzyme in a sufficiently high concentration while keeping the enzyme at work is inevitable.[6,8] *F*OYE-1 was reported to be stable at elevated temperatures,[14b] which often correlates with a higher general stability.[18,19] Thus we herein investigated the enzymatic activity and stability towards various organic solvents.

Typical solvents used in biocatalysis were analyzed in view of compatibility with the enzymatic reaction in a concentration range of 0 – 60 vol%, using both, natural and mimic cosubstrate (Fig. 2). For the NADPH data set (Fig. 2a), most solvent candidates, including ethanol, methanol, acetone, isopropanol, DMSO and THF, caused no significant loss in activity when supplemented to a final volume of 20% of the assay. Even an increase up to 120% relative activity was found in few cases; an observation which has been reported previously to some extend for other ERs.[14a,17,18] Even at 40 vol% solvent concentration the enzyme showed more than 50% relative activity for most additives, which is outstanding when compared to other ERs. Only the presence of acetonitrile led to a significant enzyme inactivation even at low concentrations. Thus, it can be concluded that *F*OYE-1 is stable against a variety of useful co-solvents and can even become more active under those conditions. Especially ethanol, acetone or isopropanol are promising candidates as they increase the relative enzymatic activity to a certain extent and can be used up to 30-40 vol% to support substrate and electron donor solubility. With respect to the use of NAD(P)H as source of electrons for ERs (*in vitro*), dehydrogenases are necessary to recycle these electron donors and these enzymes accept ethanol or isopropanol.[20] Thus the application of those co-solvents did not only support the activity of *F*OYE-1 but could also provide access to an efficient NAD(P)H recycling.

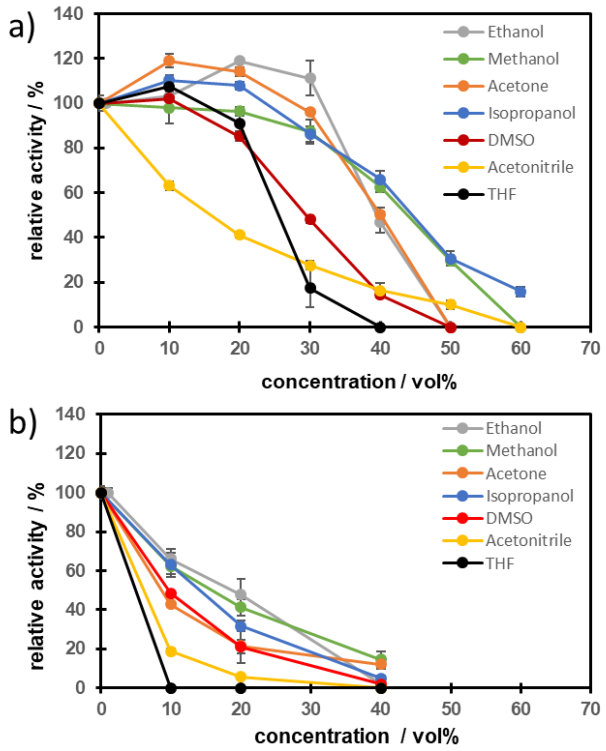

Figure 2. *F*OYE-1 activity in presence of co-solvents. The standard enzyme assay was performed while the concentration of solvents was varied, and initial rates were determined. 200 µM NADPH (a) or 1000 µM BNAH (b) served as electron donor and 1 mM *N*-methylmaleimide **2** was used as substrate. Data are shown as relative values to an enzyme assay without co-solvents (a: 100% = 140 U mg^{-1} and b: 100% = 170 U mg^{-1}).

We furthermore analyzed *F*OYE-1 activity in presence of organic solvents and BNAH as the electron donor (Fig. 2b). In all cases the enzymatic activity dropped. Especially THF and acetonitrile led to a reduced enzyme performance in terms of initial activity. Still, ethanol and methanol can be used up to 20 vol%, retaining 50% relative activity. The other tested solvents, isopropanol, acetone and DMSO, allowed a moderate enzyme performance between 10 and 20 vol% added solvent. At this point it is questionable why an exchange of electron donor led to this reduction of enzymatic activity. It should be noted that the enzyme's stability appears to be unaffected, as with NADPH as source of reducing power a much better performance was found (see also below and Fig. S4). Thus the combination of organic solvents and BNAH must cause this loss of activity and remains to be investigated in more detail. For the present study, it was crucial to show that *F*OYE-1 can work under those conditions and allows the selective conversion of a desired substrate.

For the next step, we studied the storability of the enzyme for more than 24 h in presence of co-solvents (20 vol%, Fig. S4). The enzyme is stable in 20 vol% ethanol, acetone or isopropanol for more than 24 h when kept on ice. No loss in activity was determined; in the first 2 h even an increase of activity was observed. Furthermore, the enzyme activity was studied at various temperatures (Fig. S4) and similar results as published previously were obtained.[14b] Up to 40°C, the enzyme is highly active, but at higher temperatures the activity decreases significantly.

The observation that *F*OYE-1 and other ER enzyme activity can be enhanced by adding solvents might have two reasons: *i)* increased solubility, thus availability, of (co)substrates and/or *ii)* increased flexibility of the enzyme itself. We believe the second point contributes most, as the employed (co)substrates, exclusive of BNAH, in these experiments were generally soluble in buffer (50 mM phosphate buffer). Thus, we conclude that on the one side, the presence of a co-solvent (up to 30% ethanol, acetone or isopropanol) can lead to beneficial structural changes or more mobility in the protein, most likely due to an altered solvent accessibility or a changed hydrogen bond network in the protein (backbone). On the other side this introduced flexibility might hamper the binding of nicotinamide mimics like BNAH which have *per se* a weaker binding to the active site; obvious from K_m values of 72 μM_{NADPH} *vs.* 600 μM_{BNAH}.[14b] The K_m value for BNAH even doubles in the presence of acetone (Table 3) which supports this hypothesis. Therefore, we can conclude that *F*OYE-1 is stable towards various organic solvents but those affect the performance of enzyme in dependence on the employed electron donor.

Biotransformation of (*R*)-carvone. The conversion of (*R*)-carvone **12** by means of ene-reductases is known and therefore used herein as a model reaction for an industrially relevant substrate.[2,6] In order to rule out that the applied cosubstrates NADPH or BNAH promote the direct reduction of (*R*)-carvone, respective standard assays were performed in the absence of the enzyme *F*OYE-1. Neither (*R*)-carvone reduction nor dihydrocarvone production was determined by GC-FID (data not shown).

To get a first view on the enzymatic performance in a scaled-up process, the consumption of BNAH over time was assayed (Fig. S5). Here crude extract and purified enzyme preparations were compared (each about 1 µM *F*OYE-1 as holoprotein). It was determined that 10 mM BNAH were rapidly consumed and mostly converted within 2 h. Both preparations performed similarly, with the crude extract preparation being slightly slower. However, (*R*)-carvone **12** was not fully converted at this stage (not shown). This indicates that BNAH may need to be added stepwise and a fed-batch-like biotransformation should be established.

This information was used to set up two 10 mL biotransformations of (*R*)-Carvone **12**, one with chromatographically enriched *F*OYE-1 obtained from Ni-chromatography and one containing a crude extract preparation, each with about 1 µM holoprotein. The concentration of BNAH was set to 10 mM at the beginning, and then an hourly addition of solid BNAH, corresponding to 7.5 mM, occurred to achieve maximum substrate conversion into the desired product (Fig. 3, Table S3). After 2 h, 21% conversion of **12** into (2*R*,5*R*)-dihydrocarvone was achieved with >99 % *ee* for the chromatographically enriched protein preparation. Conversion reached a maximum of 23 % (>97 % *ee* of the product) after 8h. Surprisingly, by employing the cell-free crude extract preparation, 42% conversion (>98 %*ee* of the product) were already reached within 2h. This indicates that cell-free crude extract bearing ER is sufficient to quickly produce large quantities of product in a decent

purity. However, if necessary, the one-step purification method allows to get slightly higher product enantiopurity at the price of lower conversion rates.

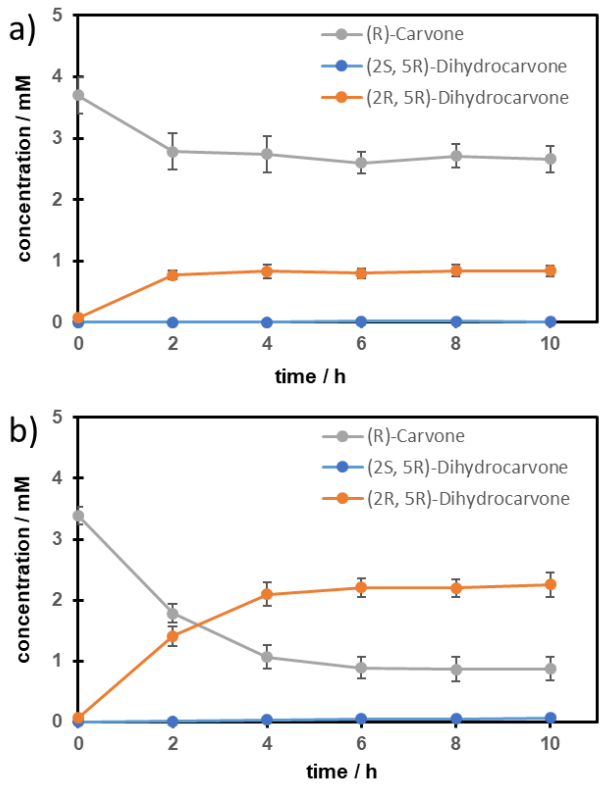

Figure 3. Biotransformation of (*R*)-carvone **12** with *F*OYE-1 with different protein preparations, 10 mL scale. For comparison a) a chromatographically enriched or b) a crude extract preparation was employed. 1 µM enzyme was applied to convert 5 mM (7.5 mg) substrate **12** while the electron donor BNAH was fed stepwise (initial 10 mM + 7.5 mM hourly in solid form). Substrate and products were analysed by chiral GC.

Finally, an experiment was performed at 750 mg-scale to show the scalability and accessibility of ER products. Thus 751 mg (*R*)-carvone **12** in 200 mL reaction volume, corresponding to 5 mmol, were converted under optimal conditions for *F*OYE-1: 15 vol% acetone in phosphate buffer at 30 °C. The enzyme was applied as crude extract preparation, comprising about 1.5 µM FMN-loaded *F*OYE-1. BNAH was provided from the beginning and fed overtime to achieve a high substrate conversion. BNAH was completely consumed at the end of the reaction. The reaction was stopped after 8 h and extracted by *n*-pentane (>99% extraction yield: 750 mg dried product/substrate mix). The actual yield and purity were determined by NMR (Fig. S7 and S8) and GC-FID. This allowed to differentiate between substrate and product (both possible diastereomers). Thus, a final extracted product of 495 mg (*2R*,*5R*)-dihydrocarvone was obtained which corresponds to 65% yield. The optical purity achieved was 95% in the final preparation and thus comparable to the small-scale biotransformation (Table S3). This shows that scale-up and changing the conditions (higher solvent concentration and using a BNAH feeding strategy) does not significantly affect the selectivity in *F*OYE-1 biocatalysis. Moreover, the bioconversion could be performed without adding an organic co-solvent: By employing pure (*R*)-carvone as second phase, conveniently serving as a reservoir for BNAH, a substrate load of 100 g/L (10 % v/v in overall volume) was achieved, resulting in similar conversion rates as before (data not shown).

Conclusions

*F*OYE-1 production is reproducible and simple to achieve. The protein can be applied as crude preparation in semi-synthetic approaches and thus chromatographic enrichments or even polishing by sophisticated chromatographic methods are avoided. The enzyme is oxygen-insensitive and accepts various nicotinamide cofactor mimics as electron donors. It is stable at elevated temperatures (30 to 40 °C) even in the presence of various organic solvents such as acetone or isopropanol (up to 30 vol%), allowing an efficient *in situ* NAD(P)H regeneration by alcohol dehydrogenases (not shown).[20] *F*OYE-1 has one of the highest ER activities reported when employing NADPH as reductant: 264 to 320 U mg^{-1} (here on maleimides) at 30 °C. Even with BNAH, an activity of 88 to 207 U mg^{-1} for the conversion of **2** was observed. Using BNAH as artificial cosubstrate somewhat limits the applicability of organic solvents due to overall lower reaction rates. Nevertheless, for experiments at various scales BNAH is still favored as a cost reduction by a factor of 50 can be achieved just by using mimetic reducing equivalents. In case of the herein performed 200 mL biotransformation this amounted to a cost of about 130€ for BNAH while NADPH would have cost 6400€ from commercial suppliers. The costs can be further reduced by synthesizing BNAH freshly, as was done here. As *F*OYE-1 showed a long lifetime in respective solvents, the reaction time can be increased to compensate lower turnover rates. Among the tested substrates, (*R*)-carvone seemed a promising candidate for the conversion into a valuable product since the production of (*2R*,*5R*)-dihydrocarvone – our model compound herein – was achieved at high conversion rates (up to 65% extracted product in 8 h by a crude preparation) and with high optical purity (>95%). To sum up, it was shown for the first time that *F*OYE-1 acts efficiently with BNAH as electron donor and is stable and active in various organic solvents. Even though the addition of co-solvents is necessary and lowers the enzyme activity when BNAH is used, this approach allows to simply convert selected substrates for biochemists, chemists and others. *F*OYE-1 can thus be considered as a valuable and easily accessible addition to the toolbox for selective hydrogenation reactions of C=C double bonds.

Experimental Section

Chemicals. NADPH and NADH were purchased from Prozomix. BNAH was freshly synthesized as described before and controlled for stability by

UV/vis spectroscopy prior to each application.[13a] All other chemicals were purchased from Sigma-Aldrich (Germany) or TCI Europe (Belgium or Germany) at highest purity available.

Enzyme production. The previously described expression vector pET_FOYE_01 was checked by sequencing with pET primers and freshly transformed into *E. coli* BL21 (DE3) for gene expression.[14b,21] A single colony was used to inoculate 50 mL pre-cultures comprising LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl per L) and appropriate antibiotics (100 µg mL^{-1} ampicillin and 50 µg mL^{-1} chloramphenicol). These were incubated at 37°C overnight and used to inoculate main cultures of each 500 mL LBNB medium (10 g tryptone, 5 g yeast extract, 29.2 g NaCl, 2 g glucose per L, and 1 mM betaine) and antibiotics in same final concentrations as mentioned above. The cultivation was routinely followed by measuring the optical density (OD) at 600 nm against a medium blank. The incubation was kept at 37°C until an OD_{600} of about 0.4 was reached, then cooled to 22°C and gene expression was induced at an OD_{600} of about 0.6 by adding IPTG (isopropyl *β-D*-1-thiogalactopyranoside: 0.1 mM final concentration). The protein production was continued overnight at these conditions and finally cells were harvested by centrifugation (4,200 x g, 40 min). The cell-pellet obtained was resuspended in phosphate buffer (50 mM Na_2HPO_4 / KH_2PO_4, pH 7.1) and stored in aliquots at -20 °C until further processing.

Enzyme purification. The cell-pellet was thawed and treated by an ultrasonic probe on ice (10 cycles of 30 s treatment at 50% duty and output by means of a probe; 60 s pause between each treatment to cool the sample; Branson Sonifier 250). DNase I (5 µL of 1 mg mL^{-1} DNase I grade II) was added to degrade the DNA prior to further processing. Then various routes were followed to prepare different purification grades of the biocatalyst: a) crude extract, b) affinity chromatographically enriched, and c) ammonium sulphate precipitation (cf. supporting information). The purity of protein samples was analysed by means of SDS-PAGE as reported earlier.[14]

a) The crude extract sample was prepared as follows: after sonication and centrifugation (13,200 rpm for 40 min at 4°C) the sample was aliquoted in 1 mL fractions and flash-frozen by liquid nitrogen. Then the crude extract preparation was stored at -20 °C.

b) The chromatographically enriched sample was prepared by means of Ni-affinity chromatography from crude extract samples as follows: Centrifugation of cell debris at 17,000 x g for 40 min at 4 °C gave a cell-free crude extract. Then the supernatant was filtered with a 0.2 µm pore-size syringe filter. Protein purification was achieved with a 5 mL HisTrap HP column using an ÄKTA device (both GE Healthcare) as described earlier.[14b] During this procedure, the removal of nonspecific proteins was achieved by rinsing first with binding buffer (10 mM Tris-HCl, 500 mM NaCl). A washing step was performed with 50 mM imidazole containing buffer (10 % of elution buffer; 10 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole). Elution of the target protein *F*OYE-1 was achieved with a linear imidazole gradient from 50 to 500 mM over 8 column volumes. Fractions containing the enzyme (monitored by the yellow color due to the presence of the flavin cofactor; can be determined at 460 nm; protein elution was also followed at 280 nm) were collected, pooled, and subsequently concentrated using an ultrafiltration device with a molecular weight cut-off of 30 kDa. Protein aliquots were stored at -20 °C in 50 mM phosphate buffer (Na_2HPO_4 / KH_2PO_4, pH 7.1) containing 50 vol% glycerol.

Protein, cofactor and activity determination. The protein concentration was measured by the Bradford method[22] using Bovine serum albumin (BSA) as standard to generate a calibration curve. Absorbance of standard and *F*OYE-1 samples was measured at 595 nm in a plate reader (Bio-Tek Instruments µQuant) against a blank of the corresponding buffer.

The loading of *F*OYE-1 with the cofactor FMN was determined by FMN quantification directly from the protein samples. A known protein dilution was prepared in a 1 mL quartz cuvette and the absorbance measured at 460 nm (extinction coefficient: 12.5 mM^{-1} cm^{-1}).[14a,17,23] The saturation of protein with FMN was calculated by comparing the protein concentration and FMN amount of the samples, respectively.

The loading of *F*OYE-1 with additional FMN was tried *via* various approaches. Firstly, either FMN was provided prior or after Ni-affinity chromatography in excess over estimated *F*OYE-1 concentration. Surplus FMN was removed by ultrafiltration as described above. However, this did not yield a fully FMN-saturated protein pool and was therefore not further investigated. In addition, the purified *F*OYE-1 was assayed for NADPH or BNAH consumption with excess FMN (10 to 70 µM, see below for assay details). The increase in rate was used as a factor representing the amount of participating active sites. Also in this case, only a partial saturation was achieved.

The ene-reductase activity assay for *F*OYE-1 was performed as described previously.[14] The assay was prepared in 1 mL quartz cuvettes and the consumption of NADPH was measured spectrophotometrically at 340 nm (extinction coefficient: 6.22 mM^{-1} cm^{-1}) (Fig. S6; example shown for different protein samples). In the case of BNAH the same procedure was followed. BNAH stocks were prepared in methanol for kinetic experiments or it was used in solid form for biotransformation studies. Extinction was measured at 340 nm (extinction coefficient: 4.75 mM^{-1} cm^{-1}) in a concentration range from 0 to 350 µM, above this concentration of BNAH we measured at 395 nm (extinction coefficient: 1.70 mM^{-1} cm^{-1}) to avoid detector saturation. The absorbance maximum of BNAH in the applied buffer was determined to be 358 nm (extinction coefficient: 6.18 mM^{-1} cm^{-1}). The assays contained a final concentration of 50 mM KH_2PO_4/Na_2HPO_4 buffer (pH 7.1), 1 mM substrate (mostly *N*-methylmaleimide **2** or *(R)*-carvone **12** used as standard) and 200 µM co-substrate NADPH if not otherwise stated. Typically, the assay was performed at 22.5 °C while all components were preheated prior measurements. A final concentration of 8.6 nM (0.375 µg mL^{-1}) holoprotein *F*OYE-1 was added to start the reaction.

The enzyme activity is expressed in U mg^{-1} which is defined as follows: 1 unit accounts for the conversion of 1 µmol substrate per minute. This was correlated to the amount of FMN-saturated *F*OYE (holoprotein) in mg.

Biotransformation. Small scale biotransformations were performed in 1 mL reaction volume in a 2 mL microreaction tube. The reactions were set up with 25 mM KH_2PO_4/Na_2HPO_4 buffer, 1.875 µM holo-enzyme, 10 mM of the respective electron donor (NAD(P)H or mimic) and 10 mM substrate. Control experiments were performed with (*R*)-carvone **12** as substrate in absence of protein. Tubes were shaken for 4 h at 50 °C at 800 rpm (thermoblock). Reactions were stopped through the addition of 500 µL ethyl acetate (containing 5 mM dodecane as an internal standard) and vortexed for 30 seconds. Afterwards, the tubes were centrifuged for 5 min at 12,000 rpm. The organic supernatant was isolated, dried with anhydrous magnesium sulfate, centrifuged and transferred into GC vials for analysis.[14a]

The biotransformation of (*R*)-carvone was performed in a final volume of 10 mL (solvent: 50 mM KH_2PO_4/Na_2HPO_4 buffer (pH 7.1) containing 15 vol% acetone). 5 mM (7.5 mg) substrate and an initial co-substrate (BNAH) concentration of 10 mM were added. To start the reaction, 1 µM *F*OYE-1 was added to the preheated solution and the mixture gently shaken at 30°C. The BNAH concentration was increased every hour by adding 7.5 mM in solid form. The formation of product was measured hourly by sampling and chiral GC/FID analysis (see below). The samples drawn (300 µl each) were treated as stated above before analysis by GC. The experiment was performed for 10 h. The final product could also be

extracted by using *n*-pentane and a subsequent concentration step by applying reduced pressure to obtain an enriched product fraction.

The same procedure was repeated at higher scale. 200 mL reaction volume in a 250 mL round-bottom flask was prepared as described above in phosphate buffer: 15 vol% acetone, 25 mM (751 mg) (*R*)-carvone, 25 mM BNAH (initial + 7.5 mM hourly), and 1.5 µM *F*OYE-1. The reaction was started by adding the enzyme (1.5 µM FMN loaded *F*OYE-1) and incubated at 30 °C (gently shaken) until the conversion stopped after about 8 h.

Product identification. In case of small scale biotransformations, the products and purity were identified by GC-FID or HPLC-UV/vis analyses as described previously.[14] Otherwise, substrate and product concentration were determined by GC-FID with a Shimadzu 2010 GC system containing a Hydrodex β-6TBDM column (Macherey-Nagel, Germany). The samples were extracted with 1:1 volume of ethyl acetate containing 1-octanol as internal standard. After extraction, the organic phase was dried with anhydrous magnesium sulfate and the supernatant placed in an analytic vial for further measurement. The column temperature was kept constant at 120 °C and the measurement took place for 10 minutes. Retention times were determined by means of authentic standards as follows: 1-octanol 3.5 min, dihydrocarvone (*R*/*S*-enantiomers) 5.2/5.7 min, and (*R*)-carvone 7.7 min.

In addition, ^{1}H NMR spectra (Bruker, Rheinstetten, Germany; DPX-200 NMR) were recorded for the product obtained from the 200 mL experiment. For this, the reaction mixture was extracted with pentane, giving a mixture of products and substrate suitable for ^{1}H NMR analysis and leaving BNAH undissolved in the reaction mixture.

(*R*)-Carvone: ^{1}H NMR (200 MHz, DMSO-d_6) δ 7.00–6.67 (m, 1H), 4.88–4.61 (m, 2H), 2.76–2.54 (m, 1H), 2.51 – 2.19 (m, 6H), 1.78–1.63 (m, 6H).

(2*R*, 5*R*)-Dihydrocarvone: ^{1}H NMR (200 MHz, DMSO-d_6) δ 4.88–4.51 (m, 2H), 2.47–2.13 (m, 4H), 2.13–1.95 (m, 1H), 1.92–1.51 (m, 5H), 1.40–1.15 (m, 1H), 0.99–0.77 (m, 3H).

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Keywords: biotransformations • oxidoreductases • old yellow enzyme • solvent stability • biocatalysis • cofactor mimics

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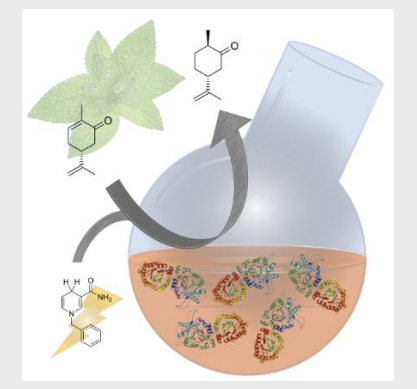
Layout 1:

FULL PAPER

Ene-reductases (ERs, OYEs) are versatile biocatalysts for the selective hydrogenation of activated C=C double bonds. Here, *F*OYE-1 was characterized with respect to solvent stability for the conversion of (*R*)-carvone towards (2*R*,5*R*)-dihydrocarvone on the expense of the synthetic nicotinamide cofactor 1-benzyl-1,4-dihydronicotinamide (BNAH). Activity is compared to the natural electron donor NAD(P)H.



Dirk Tischler, Eric Gädke, Daniel Eggerichs, Alvaro Gomez Baraibar, Carolin Mügge, Anika Scholtissek, and Caroline E. Paul*

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Applying a thermostable and organic solvent tolerant ene-reductase for the asymmetric reduction of (*R*)-carvone